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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/528,082	03/14/2005	Joseph D Mosca		5825

7590 11/30/2007

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EXAMINER	
BLANCHARD, DAVID J	

ART UNIT	PAPER NUMBER
1643	

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11/30/2007	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	10/528,082	MOSCA, JOSEPH D	
	Examiner	Art Unit	
	David J. Blanchard	1643	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *08 September 2007*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-15* is/are pending in the application.

4a) Of the above claim(s) *9-15* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-8* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☒ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *14 March 2005* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

1. Applicant's election of the invention of Group I, claims 1-8 in the reply filed on 08 September 2007 is acknowledged. Because applicant did not distinctly and specifically point out the supposed errors in the restriction requirement, the election has been treated as an election without traverse (MPEP § 818.03(a)).

2. Claims 9-15 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected invention, there being no allowable generic or linking claim.

3. Claims 1-8 are under consideration.

Specification

4. If applicant desires to claim the benefit of a prior-filed application under 35 U.S.C. 120, a specific reference to the prior-filed application in compliance with 37 CFR 1.78(a) must be included in the first sentence(s) of the specification following the title or in an application data sheet. For benefit claims under 35 U.S.C. 120, 121 or 365(c), the reference must include the relationship (i.e., continuation, divisional, or continuation-in-part) of the applications.

If the instant application is a utility or plant application filed under 35 U.S.C. 111(a) on or after November 29, 2000, the specific reference must be submitted during the pendency of the application and within the later of four months from the actual filing date of the application or sixteen months from the filing date of the prior application. If the application is a utility or plant application which entered the national stage from an international application filed on or after November 29, 2000, after compliance with 35 U.S.C. 371, the specific reference must be submitted during the pendency of the application and within the later of four months from the date on which the national stage commenced under 35 U.S.C. 371(b) or (f) or sixteen months from the filing date of the prior application. See 37 CFR 1.78(a)(2)(ii) and (a)(5)(ii). This time period is not extendable and a failure to submit the reference required by 35 U.S.C. 119(e) and/or 120, where applicable, within this time period is considered a waiver of any benefit of

such prior application(s) under 35 U.S.C. 119(e), 120, 121 and 365(c). A benefit claim filed after the required time period may be accepted if it is accompanied by a grantable petition to accept an unintentionally delayed benefit claim under 35 U.S.C. 119(e), 120, 121 and 365(c). The petition must be accompanied by (1) the reference required by 35 U.S.C. 120 or 119(e) and 37 CFR 1.78(a)(2) or (a)(5) to the prior application (unless previously submitted), (2) a surcharge under 37 CFR 1.17(t), and (3) a statement that the entire delay between the date the claim was due under 37 CFR 1.78(a)(2) or (a)(5) and the date the claim was filed was unintentional. The Director may require additional information where there is a question whether the delay was unintentional. The petition should be addressed to: Mail Stop Petition, Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

If the reference to the prior application was previously submitted within the time period set forth in 37 CFR 1.78(a), but not in the first sentence(s) of the specification or an application data sheet (ADS) as required by 37 CFR 1.78(a) (e.g., if the reference was submitted in an oath or declaration or the application transmittal letter), and the information concerning the benefit claim was recognized by the Office as shown by its inclusion on the first filing receipt, the petition under 37 CFR 1.78(a) and the surcharge under 37 CFR 1.17(t) are not required. Applicant is still required to submit the reference in compliance with 37 CFR 1.78(a) by filing an amendment to the first sentence(s) of the specification or an ADS. See MPEP § 201.11.

Claim Rejections - 35 USC § 112

5. The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

6. Claims 3-5 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 3-5 are indefinite for reciting "derived" and "tumor-derived". The claims are indefinite for reciting "derived" and "tumor-derived" as the exact meaning of the terms is not known. The term "derived" is not one which has a universally accepted meaning in the art nor is it one which has been adequately defined in the specification. The primary deficiency in the use of this phrase is the absence of an ascertainable meaning for said term. Since it is unclear how the tumor cells are to be derivatized to yield the class of derivatives referred to in the claims, there is no way for a person of skill in the art to ascribe a discrete and identifiable class of compounds to said term. In addition, since the term "derived" does not appear to be clearly defined in the specification, and the term can encompass tumor cells modified genetically by amino acid substitutions, insertions, or deletions, modified by antisense, modified by cell fusion, modified chemically or even tumor cell mimetics. In absence of a single defined art recognized meaning for the phrase and lacking a definition of the term in the specification, one of skill in the art could not determine the metes and bounds of the claims.

Claim Rejections - 35 USC § 102

7. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

8. Claims 1-7 are rejected under 35 U.S.C. 102(e) as being anticipated by Hiserodt et al (U.S. 6,277,368 B1, filed 7/24/1997).

The claims are drawn to a method for treating a cancer patient to induce an effector cell immune response or T cell immune response against cancerous cells, comprising administering tumor-derived biologically generated particles that have been

modified to mimic cells capable of presenting antigens to a mammalian immune system in an amount effective to induce an immune response against the cancerous cells to reduce the amount of cancerous cells, wherein the tumor-derived biologically generated particles are released from homologous tumor cells derived from the patient, or wherein the tumor-derived biologically generated particles are released from matched major histocompatibility complex containing tumor cells, or wherein the tumor-derived biologically generated particles are released from non-homologous tumor cell lines containing one or more matched human leukocyte antigens, or wherein the particles are generated as virus-like particles or inactivated intact virus particles.

Hiserodt et al teach a method of treating cancer in a subject comprising inducing a cellular immune response involving T cells (i.e., "effector cell immune response") against cancer cells comprising administering tumor cells modified to express a cytokine and optionally altered to express additional cytokines, additional tumor-associated antigens, additional cell-surface molecules, such as adhesion molecules like ICAM-1, histocompatability antigens, or co-stimulation markers like B7-1 or B7-2 and the tumor cells may be autologous or allogeneic, are inactivated and wherein the cytokine-expressing cells are produced using a viral vector such as adenoviral and retroviral vectors (i.e., "virus-like particles" and "inactivated intact virus particles") (see entire document, particularly cols. 7-10, 15-19, and 21-23).

Thus, Hiserodt et al anticipates the claims.

Conclusion

9. The prior art made of record and not relied upon is considered pertinent to applicant's disclosure.

Jaffee et al. Methods: A Companion to methods in Enzymology 12:143-153, 1997. Jaffee et al teach genetically modified cell-based immunotherapy for eliciting an antitumor immune response, including modifying autologous and allogeneic tumor cells to secrete cytokines using viral vectors.

Jaffee et al. U.S. Patent 5,985,290, issued 11/16/1999. Jaffee et al teach methods of treating cancer comprising administering genetically modified allogeneic, but not MHC-matched tumor cell lines that produce increased level of cytokine relative to the unmodified tumor cell line.

10. An examination of this application reveals that applicant is unfamiliar with patent prosecution procedure. While an inventor may prosecute the application, lack of skill in this field usually acts as a liability in affording the maximum protection for the invention disclosed. Applicant is advised to secure the services of a registered patent attorney or agent to prosecute the application, since the value of a patent is largely dependent upon skilled preparation and prosecution. The Office cannot aid in selecting an attorney or agent.

A listing of registered patent attorneys and agents is available on the USPTO Internet web site http://www.uspto.gov in the Site Index under "Attorney and Agent Roster." Applicants may also obtain a list of registered patent attorneys and agents located in their area by writing to the Mail Stop OED, Director of the U. S. Patent and Trademark Office, PO Box 1450, Alexandria, VA 22313-1450

Any inquiry concerning this communication or earlier communications from the examiner should be directed to David J. Blanchard whose telephone number is (571) 272-0827. The examiner can normally be reached at Monday through Friday from 8:00 AM to 6:00 PM, with alternate Fridays off. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Larry Helms, can be reached at (571) 272-0832.

The official fax number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you

have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

/David J. Blanchard/
Primary Examiner, A.U. 1643

Notice of References Cited	Application/Control No. 10/528,082	Applicant(s)/Patent Under Reexamination MOSCA, JOSEPH D	
	Examiner David J. Blanchard	Art Unit 1643	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-5,985,290	11-1999	Jaffee et al.	-----
*	B	US-6,277,368 B1	08-2001	Hiserodt et al.	-----
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Jaffee et al. Methods: A Companion to methods in Enzymology 12(2):143-153, 1997.
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

METHODS: A Companion to Methods in Enzymology 12, 143–153 (1997)
Article No. ME970463

Considerations for the Clinical Development of Cytokine Gene-Transduced Tumor Cell Vaccines

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In preclinical models, tumor cells genetically altered to secrete cytokines or express costimulatory molecules can generate systemic antitumor immunity. In some studies, these tumor vaccines have been shown to eradicate micrometastases. These results have led to the initiation of numerous phase I clinical trials employing either genetically modified or allogenic tumor vaccines. This article addresses a number of issues related to the clinical development of cytokine gene-transduced tumor cell vaccines including: (1) the production of cytokine-secreting tumor vaccines; and (2) the preclinical feasibility and toxicity studies required for testing these vaccines in patients with cancer. © 1997 Academic Press

Increasing evidence supports the notion that all tumors possess tumor-specific antigens capable of being recognized by antigen-specific T cells. These tumor-specific neoantigens most likely either are the consequence of novel amino acid sequences generated by mutations or rearrangements or, alternatively, derive from genes that are silent in normal cells but become activated in the tumor cell. The fundamental question still facing tumor immunologists is how can the immune system be activated to recognize and eradicate cells expressing these tumor antigens? A recently developed vaccination strategy, employing tumor cells genetically altered to secrete cytokines in a paracrine fashion, has begun to shed light on the answer to this important question. This article provides an overview on the current knowledge of the mechanism of antitumor immune responses and focuses on the methods required for further study of immune responses generated by cytokine-secreting tumor vaccines.

RATIONALE FOR GENETICALLY MODIFIED, CELL-BASED IMMUNOTHERAPY

The rationale for genetically altered tumor vaccine-based immunotherapy of established cancer depends on the existence of antigens within tumors that can be recognized as foreign by the host immune response. It is the tremendous diversity of the T- and B-cell receptors that endows the immune system with the ability to distinguish fine antigenic differences among cells. For the generation of an antitumor immune response, two criteria must be fulfilled. First, the tumor must present recently developed antigens or neoepitopes not found on normal cells. Second, the immune system must be appropriately activated to respond to these recently developed antigens. The classic studies of Boon and colleagues (1, 2) have demonstrated that tumor antigens recognized by T cells fall into one of three categories: (1) recently developed peptide sequences generated by point mutations in genes encoding various cellular proteins, (2) examples in which the gene encoding the tumor antigen is identical to the germline sequence, but is not expressed in any normal tissues, or (3) one example in which the gene encoding the tumor antigen is a highly specific differentiation an-

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tigen. As a consequence, the immune system need not be tolerized to the gene during development and peptides derived from the nonmutated form can serve as tumor-specific antigens. The MAGE-1 gene encodes a human melanoma antigen, which was the first human antigen identified. The MAGE-1 antigen falls into the second category of tumor antigens and is expressed in as many as 50–60% of human melanomas (2). The tyrosinase protein is an example of a differentiation antigen that encodes for another human melanoma antigen (3). This antigen was found to be expressed by 39 of 39 fresh melanoma samples tested and was also overexpressed relative to normal skin samples. These antigens, plus an additional set of differentiation antigens more recently identified in human melanomas (reviewed in 4), have indeed renewed faith in the concept of common sets of tumor-specific antigens, thereby allowing for the possibility of specific antigen or peptide-based vaccination strategies. But until more common tumor antigens have been identified at the genetic level, and the prevalence and biorelevance of these antigens have been assessed, an individual's tumor is the only source of tumor antigens for vaccination at this time.

CELL-BASED IMMUNOTHERAPY: AN APPROACH TO AUGMENTING THE ANTITUMOR IMMUNE RESPONSE

Irradiated Tumor Cells Mixed with Adjuvants

The fundamental question still facing tumor immunologists is how can the immune system be activated to recognize and eradicate cells expressing tumor antigens? As early as 1893, a New York surgeon, William Coley, postulated that the injection of tumors with pyogenic bacterial extracts might nonspecifically stimulate the immune system in a fashion that ultimately enhances immune responses specific for the tumor. Since then, many investigators have tested this concept of inducing antitumor immune responses by co-injection of tumor cells with bacterially derived products as adjuvants to induce immunity against cancers. The bacilli Calmette-Guérin (BCG) and *Corynebacterium parvum* (*C. parvum*) are the two most commonly studied bacterial adjuvants, both in animal models (5–9) and in patient trials (10–13). The numerous studies employing murine models of cancer have resulted in the following observations: (1) Both BCG and *C. parvum* have appeared to either inhibit or slow the rate of growth of some murine tumors, usually when administered local to the challenge tumor; (2) Administration of these adjuvants systemically (intravenously or intraperitoneally), at a different site than local to the tumor, usually does not affect the growth rate of that tumor; (3) The tumor inhibitory effects of both BCG and *C. parvum* have been shown to be dose dependent; (4) Combination of these adjuvants with systemic chemotherapy has enhanced the antitumor response; and (5) Rarely will the local administration of these adjuvants mixed with the tumor result in eradication of distant tumor deposits. The results from patient trials have also been variable and usually disappointing. As was seen in the murine tumor models, systemic administration of the adjuvant occasionally increased overall survival (9), but did not show a durable antitumor effect (10, 11). However, most other studies have not compared parameters in a randomized fashion; this, together with the uncharacterized nature of many adjuvants, has made it difficult to determine which approach generates maximal immune responses. Furthermore, in humans, melanoma and renal cell carcinoma, the two cancers in which adjuvants have most often been studied, are notable for occasional spontaneous remissions that appear to be immunologically mediated. They may therefore represent cases in which the tumor and immune system are somehow balanced, and even relatively inefficient strategies for activation of immune responses may tip the balance in favor of the immune system over the tumor. However, in one prospectively randomized trial of patients with minimal residual (Duke's B2 and C) colon cancer in which patients received autologous, irradiated tumor cells mixed with the adjuvant, a prolonged disease-free and overall survival was observed (12). Thus, it seems possible to generate antitumor immune responses using irradiated tumor cells mixed locally with adjuvants as vaccines. Although these responses are usually weak, and therefore ineffective against the majority of established tumors, these early studies established a basis for employing immune modulators in a paracrine fashion to generate antitumor immune responses.

TUMOR CELLS GENETICALLY ALTERED TO EXPRESS FOREIGN GENES

Recently, there has been renewed interest in active immunotherapy approaches using genetically

altered tumors. The first studies demonstrating enhanced immunogenicity of genetically altered tumors were performed 25 years ago, starting with Lindenman and Klein (14), who showed that vaccination with influenza virus-infected tumor cell lysates generated enhanced systemic immune responses against a challenge with the original wild-type tumor cells. Furthermore, these early studies showed that nonvirally infected tumor cell lysates, or tumor cell lysates mixed with the same virus, are not immunogenic and cannot elicit a systemic immune response against challenge with the parental tumor cells. Because adequate immunization against the tumor required that the tumor cells be infected with the virus, Lindenman and colleagues hypothesized that weak antigens derived from the tumor cells might become associated with or incorporated into the virus and subsequently become potent immunogens. However, based on what we have learned since then about immune responses, the enhanced immune response generated by the virally infected tumor cells was probably the result of high viral protein expression and subsequent availability of both MHC class I and II antigenic epitopes required for priming the $CD4^+$ and $CD8^+$ T-cell arms of the immune system. The antitumor response that was generated in addition to the antiviral response probably occurred as a consequence of the expression of tumor antigens at the same site as the viral antigens. The studies of Lindenmann and colleagues therefore confirmed the importance of employing immune modulators in a paracrine fashion to generate antitumor immune responses.

As newer techniques of gene transfer have been developed, infection with infectious virus has been replaced with specific gene transfer in an attempt to more carefully regulate the nature of the genetic alteration in the tumor. Fearon and colleagues (15) used direct gene transfer to introduce the influenza HA gene into BALB/c-derived CT26 colon tumor cells, thereby resulting in the expression of a viral antigen on the tumor's cell surface. This approach, followed by repeated FACS sorting of high-expressing variants, resulted in transfectants that were rejected by BALB/c mice at doses 100 times greater than the parental tumor. Again, these HA transfectants induced a systemic immune response against challenge with parental CT26 tumors. While only high HA expressors were able to be rejected by syngeneic animals and induce systemic immune responses against the original tumor, there was significant discrepancy between different subclones of HA transfectants expressing similar HA levels, suggesting that factors other than simply HA expression contributed to immunogenicity of the tumor. Unfortunately, this approach has not been found to consistently generate adequate antitumor responses in other tumor systems.

TUMOR CELLS GENETICALLY ALTERED TO SECRETE CYTOKINES IN A PARACRINE FASHION

Animal Models

Most recently, there has been intense interest in the study of immune responses generated by tumor cells engineered to secrete various cytokines. This strategy does not involve inducing the expression of any foreign genes in tumor cells, but rather seeks to locally alter the immunological environment of the tumor cell so as to either enhance antigen presentation of tumor-specific antigens to the immune system or enhance the activation of tumor-specific lymphocytes. As mentioned above, one of the most important concepts underlying the use of cytokine gene-transduced tumor cells is that the cytokine is produced at very high concentrations local to the tumor. Systemic concentrations are generally quite low. This paracrine physiology much more closely mimics the natural biology of cytokine action than does the systemic (intravenous) administration of recombinant cytokines. Many cytokine genes have been introduced into tumor cells with varying effects on both tumorigenicity and immunogenicity. Some of these cytokines, when produced by tumors, induce a local inflammatory response that results in elimination of the injected tumor. This local inflammatory response is most often predominately dependent on leukocytes other than classical T cells. These systems have been used to uncover *in vivo* effects of cytokines that result in activation of tumoricidal potential by various types of leukocytes.

This new tumor vaccine approach using genetically altered autologous tumor cells to secrete local concentrations of cytokines has been best developed in murine models (16–24). In some of these models, lymphokine gene-transduced tumor cells have been shown to generate a local, tumor-specific immune response when administered as a subcutaneous vaccination (16–18, 20–24). In addition to rejecting the genetically modified tumor cells, vaccinated animals

may develop a T-cell-dependent systemic immunity, which in some cases can cure micrometastases established prior to treatment with the genetically altered tumor cells (16–18, 22–24). In all cases in which systemic immunity against wild-type tumor challenge has been analyzed, it is mediated by T cells. Given the number of studies done to date with cytokine-transduced tumor cells, it is not surprising that variable results have been seen when different tumor systems are analyzed. Additional variables to the cytokines employed include cell dose, level of cytokine expression, location of immunization and challenge sites, and vaccination schedule. All of these parameters critically affect vaccine efficacy for cytokine gene-transduced tumor cells.

Given the large number of potential cytokine genes in the armamentarium, and the technical difficulties in transducing human tumor cells to make vaccines, it is critical that they be compared for efficacy. Also, given that most mouse tumors show significant immunogenicity when simply irradiated, identification of genes that truly enhance the immunogenicity of a tumor above that of irradiated wild-type cells is important. The first study that directly compared multiple cytokine and other genes in murine tumor models used a highly transmissible defective retroviral vector. This study demonstrated that, in a number of poorly and moderately immunogenic tumors, immunization with granulocyte–macrophage colony-stimulating factor (GM-CSF)-transduced tumors produced the greatest degree of systemic immunity, which was enhanced relative to irradiated nontransduced tumors (22). Immunity was dependent on both $CD4^+$ and $CD8^+$ T cells, despite the fact that the tumors were MHC class II^-. The potency of GM-CSF's effect locally may relate to its unique ability in promoting the differentiation of hematopoietic precursors to dendritic cells, which are the most potent antigen-presenting cells for helper T cells (25, 26).

Developing Human Cytokine-Secreting Tumor Vaccines

While the recent preclinical studies performed in mouse tumor models are indeed paving the way for clinical trials with genetically engineered tumor vaccines, a number of critical considerations remain that will have significant impact on whether this new immunotherapy approach will translate into true clinical benefit for human cancer patients. First, most human tumors are difficult to establish as cell lines, even in the short-term. Yet, until cellular tumor antigens are identified, autologous cell lines will need to be established from each patient to be treated, as a means of providing tumor antigens at the site of activation of the immune system. Even in cases where subselection of continuously growing tumor lines can be achieved, there is a high likelihood that after extended passage, the antigenic composition will change significantly relative to the original primary tumor from which the tumor line originated. Second, high-efficiency gene transfer systems are needed to genetically alter these primary tumor lines. Ideally, high-efficiency gene transfer would obviate the need for co-introduction of a selection marker, thereby minimizing the level of *in vitro* passage and maximally preserving the original antigenic composition of the explanted primary tumor.

Defective retroviral vector systems are unquestionably the gold standard for high-efficiency gene transfer in the initial stages of human gene therapy. These vectors have the potential for being free of helper virus and therefore extremely safe. Nonetheless, they require at least one cell cycle for integration and gene expression to occur. Early generations of retroviral vectors have been notoriously inconsistent in their gene transfer efficiency as well as in consistency of gene expression. Recently, newer generations of retroviral vectors that have taken advantage of modifications in the intronic gag sequences as well as the positioning of the start sites of the inserted gene appear to have successfully circumvented a number of these problems and allowed efficient gene transfer into primary human tumor explants in the absence of selection (27). Other gene-transfer systems such as adenovirus-based as well as nonviral systems including liposomes are currently under evaluation, although their ultimate usefulness in clinical settings remains to be determined.

METHODS FOR PREPARING CYTOKINE-SECRETING TUMOR VACCINES

Choice of Method of Gene Transfer

Gene transfer into tumor cells can be accomplished by a variety of methods involving either naked DNA or the use of viral vectors. Methods to transfer naked DNA include: (1) coprecipitation with calcium phosphate (28); (2) the use of electroporation, which exposes cells to rapid pulses of high-

voltage current, thereby providing a physically induced opening in the cell membrane for entry of DNA (29); (3) direct introduction of DNA into cells by microinjection (30); and (4) encapsidation of DNA into liposomes (31). Use of any of these methods will often result in the introduction of multiple copies of the cytokine gene randomly into the host cell's genome. Several of these methods can result in transient expression of the gene (for 24–72 h) by as many as 50% of the cells in the transfected population, because the transfected DNA can exist free in the cell nucleus for a short time (32). However, stable gene expression, which requires integration of the transfected DNA into the host's genome, usually occurs in much less than 1% of the cells within the population undergoing transfection. To achieve stable integration and expression of the DNA into a high proportion of the cell population, it is often necessary to select for the minority of cells in the transfected population that have successfully retained the foreign DNA. This can be accomplished by cotransfecting DNA that encodes for a selectable marker, which will allow cells expressing its product to survive in growth media that contains a substrate for the gene's product that is normally toxic to most mammalian cells. In this way, *in vitro* selection of those cells that have successfully incorporated the transferred DNA can be accomplished. However, although *in vitro* selection will enhance the number of cytokine-secreting tumor cells in the cell population to nearly 100%, it is at the theoretical expense of antigen expression loss among that tumor cell population. In theory, loss of particular antigenic populations of tumor cells will decrease the effectiveness of the vaccine. Detailed protocols employing these methods can be found elsewhere (33).

Currently, the most efficient method of stable gene delivery into mammalian cells is through the use of viral vectors, which infect their target cell by binding specific cell surface receptors. Most viral vectors are constructed so that they contain the sequences encoding for the expression of the gene and all of the genetic signals including the promoters, enhancers, splicing signals, and signals for polyadenylation of RNA transcripts, all of which are necessary for the transcription and ultimate translation of the inserted cytokine gene sequences. Often, the vectors will also contain selectable markers (34). Potential adverse consequences following viral infection can include: (1) damage or death to the host cell; (2) the activation of other latent viruses integrated into the host's genome; (3) the activation of silent host genes such as protooncogenes; or (4) transformation of the defective viral vector from replication incompetent to replication competent by recombination with host gene sequences. All of these possibilities should be considered in choosing a viral vector system for gene transfer.

Retroviruses have been the most commonly employed vectors for the preparation of cytokine-secreting tumor vaccines, for study in murine models, and in human vaccine therapy trials. There are at least two reasons for this. First, as mentioned above, retroviruses usually do not enter into a lytic cycle of viral replication and therefore do not kill their host cell soon after viral infection. Second, retroviruses can infect most mammalian cells and integrate into the host genome, which is a critical requirement for efficient gene transfer and expression in a stable and heritable fashion. Most of the retroviral vectors employed in cytokine-secreting tumor vaccine studies have been developed from a murine retrovirus. A key feature of these retroviral vectors is their incompetence to replicate following transduction into the host cell (34–39).

The use of these high-efficiency retroviral vectors to carry cytokine genes into tumor cells has a number of specific advantages: (1) The transduction is rapid and selection is unnecessary. Subcloning experiments documented that nearly 100% efficiency is achieved in a 4-h infection followed by 48 h culture to allow for integration of the viral vector into the host cell's genome. (2) The absence of selection avoids the potential problem that selected subclones may display immunologic and biologic properties different from those of the original tumor cell population. (3) Using retroviral supernatants from different cytokine producer cell lines, combinations of cytokines can easily be simultaneously introduced into the same cell. However, there are also several potential disadvantages to the use of this vector system. First, entry into the cell is dependent on the expression of specific viral receptors on the host's cell surface. Because the identity of most retroviral receptors is still unknown, it is difficult to determine whether a particular host cell expresses these receptors and to what extent these receptors are expressed. Second, efficient retroviral gene transfer requires that the host cell proliferates for integration of the viral genes into the host cell's genome and for expression of the transferred gene. Third, retroviruses that contain amphotropic envelop genes are difficult to purify, concentrate, or store for long periods of time, because of their fragility. Their fragility

is probably manifested by the loss of their env gene product, which ultimately translates into decreased infectivity. Fourth, there is the theoretical concern that retroviral integration may lead to insertional mutagenesis because the virus integrates into random sites in the host's genome and may interrupt vital cellular genes or insert retroviral regulatory sequences that modify the expression of nearby cellular genes. Finally, helper virus production by the retroviral packaging line has occurred when employing some of the earlier generations of these packaging lines. This potential problem is the major concern when choosing a viral vector to employ for the production of genetically altered tumor vaccines for clinical trials. Helper virus detection has not been reported to have occurred in any of the clinical trials employing retroviral vectors thus far, nor in most of the animal studies designed to test the safety of these vectors. However, in one study in which immunosuppressed monkeys purposefully received high concentrations of replication-competent retroviruses, lymphomas developed (40), illustrating the potential seriousness of helper virus contamination.

In addition to retroviruses, other viral and nonviral gene transfer vectors are beginning to be employed to generate cytokine gene-transduced tumor vaccines. Adenoviral vectors offer the particular advantage of high titer and therefore high-efficiency gene transfer. Under optimized conditions, adenoviral packaging lines such as 293 can produce titers of 10^{12} particles/ml compared with 10^6 particles/ml for retroviruses (41). The major limitation of adenoviral vectors for many gene therapy approaches is that, because it does not integrate into the genome of the transduced cell, expression is lost after 1–4 weeks. However, because cytokine gene-transduced tumors are eliminated within 1 week of injection by the combination of irradiation and the local paracrine cytokine-dependent inflammatory response, the limited duration of expression of adenovirally transferred genes is still quite adequate for this particular type of vaccination approach.

DESIGNING *IN VIVO* STUDIES

Two basic types of *in vitro* assays are used to evaluate vaccine strategies employing cytokine-secreting tumor cells in mice: the protection and the cure assay. Both assays can be performed in a variety of ways, and it is best to optimize the following parameters for each tumor system that is studied. First, immunization may be accomplished by one of several routes of vaccine administration, including subcutaneous, intradermal, intraperitoneal, and intravenous administration of the vaccine cells. The optimal route of administration may depend on the histologic type of the tumor, the availability of an adequate vaccination space (for example, mice have a small dermis that is difficult to inject with large numbers of vaccine cells), or the number of regional lymph nodes accessible to the vaccine site. Second, effective immunization may be accomplished over a range of vaccine cells, against a range of parental challenge or existing tumor deposits. These ranges should be determined for each tumor system studied. Third, the cytokine-secreting vaccine should be compared to wild-type irradiated vaccine cells, since many experimental animal tumors become immunogenic following irradiation or other means of tumor cell inactivation. It is still often possible to study immunogenic tumors since the magnitude of priming may be less for the wild-type irradiated tumor cells compared with the cytokine-transduced tumor cells. However, this magnitude difference should be characterized to guide the design of further studies with that particular tumor system.

The protection assay is the best method for the initial screening of priming of the immune response against a tumor by different cytokine-secreting tumor vaccines. In particular, this assay provides a sensitive method for studying the magnitude of priming that has resulted following vaccination. Because the challenge antigen load is given following vaccination, and the volume of tumor can be controlled, even a low magnitude of priming can be detected, and small differences in priming between different vaccines can be observed. In contrast, cure experiments are best employed to study clinically relevant antitumor immune effects. In these studies, cytokine vaccines can be evaluated for their ability to impact on established cancer.

CONSIDERATIONS FOR IMMUNE MONITORING OF VACCINE RESPONSE

1. Histologic Evaluation of Vaccine and Challenge Sites

One of the most informative studies that can be performed on *in vitro* studies of cytokine-transduced

tumor vaccines is biopsy of the vaccine and challenge sites, at several time points following vaccination and challenge. Histologic evaluation of the vaccine and challenge sites can provide information on the type of immune cells that infiltrate each site, as well as the time course of this infiltration. Biopsies of both the vaccine and the challenge sites should be performed because it is quite likely that different arms of the immune response are involved in priming the initial antitumor immune response versus effecting the antitumor response. In addition, biopsies over a time course can provide valuable information concerning the kinetics of the priming response.

2. Lymph Node Biopsy

Biopsy and histologic evaluation of the regional lymph nodes can reveal the local areas within the nodes where immune cell priming may be occurring. This information would be helpful in hypothesizing which arm of the immune response is predominantly involved in the priming event. It may also provide information concerning the kinetics of the priming response. In addition, lymphocytes isolated from regional lymph nodes can be studied for tumor-specific lymphocyte activity in *in vitro* assays.

3. *In vitro* Assays

Many preclinical murine studies have shown that the immune response generated by autologous tumor vaccine cells genetically altered to secrete GM-CSF induces both a $CD4^+$ and a $CD8^+$ T-cell response (16–20, 22). Evidence for this comes from *in vitro* T-cell subset depletion studies in which mice were depleted of $CD4^+$, $CD8^+$, or NK cells by the administration of blocking antibodies and subsequently were vaccinated with irradiated cytokine-secreting tumor cells. In one study, $CD4^+$ and $CD8^+$ T cells were required early during the initial priming of the response and at the effector stage of the immune response (22). Therefore, both subsets of lymphocytes may play important roles at both the effector and the priming phase of the antitumor immune response.

There are several assays that are commonly employed to detect tumor-specific T cells, some of which have been shown to detect T-cell responses in preclinical models, but none of which have been proven to be of value in monitoring a patient's immune response to cancer. Even in murine models, it has been difficult to employ these assays to estimate the magnitude of the immune response generated. It is unclear whether the problem is a lack of sensitivity of the currently employed assays or the lack of generation of an antitumor immune response by the therapy that was administrated to the patient, or both.

There are several problems with the ^{51}Cr release assay as a means of measuring relevant CD8-dependent antitumor responses. First, although it is routinely possible to detect CTL in mice treated with cytokine-secreting tumor vaccines, quantitative measurements of this activity do not correlate with antitumor effects. CTL assays are best interpreted as an all-or-none response. Second, the sensitivity of the assay for detection of CTL activity is not well defined. Third, nonspecific lysis often masks the specific lysis. This can be overcome by using unlabeled, NK-specific target cells as competitive inhibitors in the lysis assay (42). Fourth, CTL assays are dependent on the operator and phase of the moon. Finally, *in vitro* tumor target cell lysis may be an artificial readout of T-cell activation. Specifically, lysis of the target tumor cell by the already activated T cell requires that at least three conditions be met. First, the T-cell receptor must engage the MHC class I molecule/antigen complex on the target cell's surface. Second, the T cell must release TNF and other target lysis factors for target killing to occur. Third, these factors need to be able to lyse the target cell. It is possible that the ^{51}Cr release assay underestimates the number of activated T cells generated by the tumor vaccine because lysis is target cell dependent, rather than T cell dependent. Thus, a comparison of this assay with other T-cell assays is necessary.

Advances in T cell cloning techniques have resulted in the ability to analyze the functional heterogeneity of lymphocyte populations. Analysis is usually performed by measuring the lytic activity of lymphocyte clones derived from limiting dilutions of bulk lymphocyte cultures against target tumor cells. This approach was recently employed by Coulie and colleagues to provide frequency estimates of tumor-specific CTL precursors among autologous peripheral blood lymphocytes of melanoma patients (43). In the seven patients with malignant melanoma that were evaluated, an estimated CTL precursor frequency between 1/900 and 1/33,000 was demonstrated. If this method can reliably measure specific T-cell frequency, it will provide a suitable and easy way of monitoring frequencies of CTL in peripheral blood of the same patient at different stages of vaccine therapy. Unfortunately, this assay is also complicated by the significant number of nonspecific

NK-like clones that grow out and possibly mask tumor-specific T-cell activity.

As mentioned earlier, preclinical data suggest that cells other than CTL play a critical role in generating an antitumor immune response. There are also some human data to support this. First, vaccine studies in patients, which usually employ autologous irradiated tumor cells with or without an adjuvant, have occasionally described the return of delayed type hypersensitivity reactions to the autologous tumor cells following vaccination. Second, in one human study designed to evaluate *in vitro* helper T-cell responses, specific antitumor reactivity was detected in tumor infiltrating lymphocytes (TIL) of patients with breast cancer, when cytokine secretion in response to coculture with autologous tumor cells was the parameter measured (44). In this study, TIL from 3 of 11 patients specifically secreted GM-CSF, TNF-α and interferon-γ when stimulated by autologous tumor and not by a panel of five allogeneic breast cancers. Secretion of IL-4, IL-6, and IL-1b were not found to be secreted by these TIL. In contrast, a previous evaluation of breast cancer TIL using CTL lysis as a readout failed to show tumor-specific activity (45). Interestingly, phenotypic analysis of the cytokine-secreting TIL isolated from all three patients revealed that at least 56% of the T cells were $CD4^+$, and in two of the three cases, 96% or greater were $CD4^+$. This provides evidence that $CD4^+$ T cells can specifically recognize autologous tumor and that this activity can be measured by cytokine secretion. In addition, this supports the need to identify other components of the immune response that may be more sensitive or accurate measures of the specific antitumor response.

CONSIDERATIONS FOR PRECLINICAL EVALUATION OF THE VACCINE RESPONSE

The success of cytokine-secreting tumor vaccines in murine models of cancer have led to the initiation of clinical trials in patients. However, many questions concerning the mechanism of activation of the antitumor immunity are still unanswered. Murine systems can continue to provide suitable models for studying this problem. In addition, these preclinical models can be employed to guide the design of the first generation of phase I clinical trials. In particular, they can be used to study critical parameters affecting vaccine efficacy, such as levels of cytokine expression, location and schedule of immunization, and range of vaccination dose, as well to predict local and systemic toxicities from the vaccine.

1. Vaccine Schedule and Routes of Administration

Both the route of administration and the vaccine schedule employed are crucial parameters that may differ for different murine tumor systems. For the treatment of solid tumor malignancies in the mouse, the subcutaneous space appears to provide an adequate area for the initial priming of immune responses, because this area has adequate blood supply and is near the lower epidermis where potential antigen-presenting cells, in particular dendritic cells, reside. The dermis is an area that should be considered in designing patient trials for the same reason, although, in mice, this area is too small to adequately access for vaccination. Consideration should be given to intraperitoneal vaccination, particularly for tumors that often metastasize to that area (ovarian cancer is the best example). Intravenous vaccination should be considered for vaccination against hematologic malignancies. An evaluation of the optimal vaccine schedule, both in terms of the number of simultaneous vaccinations and the number and schedule of repeat vaccinations, should also be performed. For example, priming of one lymph node region at a time may be less effective than priming multiple lymph node regions simultaneously. In addition, repeated weekly, biweekly, or monthly vaccinations may be more effective than a single vaccination dose.

2. Vaccination Dose

In determining the optimal vaccine dose for augmenting antitumor immune responses, two parameters need to be considered: (1) the threshold concentration of cytokine required and (2) the concentration of antigen required. Dilution studies, where the total number of cytokine-secreting tumor cells is serially decreased while maintaining the total number of antigen-expressing cells constant (by dosing in wild-type irradiated tumor), can be performed to address these parameters (46).

3. Local and Systemic Toxicity Evaluation

Toxicity evaluation in preclinical models is critical for designing phase I clinical trials because it can help to predict potential serious side effects in patients. There are two major categories of toxicities that might be expected from cytokine-secreting tu-

mor vaccines. The first category is toxicity that is the result of systemic levels of the cytokine. There are data in the literature for some cytokines that have been administered to patients, usually intravenously or subcutaneously, that may help to predict the plasma levels that may result in significant side effects. Pharmacokinetic studies can easily be performed to test for systemic levels of cytokines that result from subcutaneous or intradermal vaccination. Plasma levels of cytokine can be measured over a time course following vaccination to predict the peak time and peak concentrations of the cytokine following administration of the vaccine. The second category is autoimmune toxicity that is the result of the activation of immune responses against self antigens. This type of toxicity may result because the tumor cells themselves also express antigens normally expressed by the nonmalignant cell of origin or because normal cells were isolated at the time of excision of the tumor mass and continue to contaminate the cultured tumor cells during vaccine preparation. Unfortunately, mice are not the best models for studying autoimmune disease in general. However, it is possible to set up studies that attempt to induce autoimmunity under extreme cases of self-antigen presentation.

There are several important safety issues to be aware of when designing clinical trials for patients. The first issue is helper virus contamination. This issue is a very serious one and of greater concern when producing upscaled batches of retroviral supernatants for the trial. However, a detailed approach to screening lots of producer lines and retroviral supernatants is beyond the scope of this article. But a second safety issue concerns reinjecting live vaccine cells back into the patient and risking new tumor growth. This problem can easily be avoided by evaluating methods of inactivating the vaccine cells prior to reinjection. Several studies have shown that it is possible to lethally irradiate cytokine-secreting vaccine cells prior to reinjection, without loss of cytokine expression (22, 27). Each histologic tumor type will require different doses of gamma irradiation. It is therefore important to evaluate optimal doses of irradiation that will result in loss of proliferation without loss of short-term cytokine expression. *In vitro* studies from our laboratory have shown that lethally irradiated, cytokine-secreting murine and human tumor cell lines will continue to secrete adequate levels of cytokine for 7 days following irradiation, before the concentration of cytokine secreted begins to decline. Minimal cytokine production is usually detected 2 weeks following irradiation (47).

AUTOLOGOUS VS ALLOGENEIC VACCINES

Clinical vaccine trials employing *ex vivo* transduced tumor cells can be divided into two general types—transduction of autologous tumor cells or use of a transduced allogeneic tumor line. The autologous tumor vaccine strategies require that either the patient's primary tumor or a metatastic site is surgically excised followed by introduction of the gene *in vitro*. This strategy has the advantage that an individual's own tumor cells have the greatest likelihood of possessing the relevant tumor antigens expressed by their metastases (against which the immunologic response must be directed). Since all metastases in an individual arise from the primary tumor, the ideal vaccine would utilize a mixture of all the cells from the primary tumor, thereby providing the greatest chance to vaccinate against the spectrum of relevant tumor antigens. As discussed above, most of the gene transfer techniques require at least short-term culture tumor cells, during which time subsection of tumor cells and loss of antigens may occur. Ideal gene transfer systems utilize high-efficiency vectors and minimize the number of *in vitro* passages (48).

An example of one trial utilizing transduced autologous tumor vaccines has been initiated for patients with stage IIIb or IV renal cell carcinoma (49). Patients undergo nephrectomy to obtain the primary tumor for vaccine production. The primary tumor was chosen as the source of tumor cells from which to produce the vaccine because of the theoretical concern that different metastases, each of which probably arises from a different clone of the primary tumor, express a different set of tumor-specific antigens, whereas the primary tumor is most likely to express the entire range of tumor antigens found on all of the distant metastases. Following surgery, genetic modification of the renal tumor cells is performed using the Moloney-based MFG retroviral vector containing the human GM-CSF gene following initial expansion of the enzymatically dissociated tumor cells. Once expansion and transduction are completed, the vaccine cells are gamma-irradiated with 15,000 rad and frozen in liquid nitrogen until the day the patient is to be treated.

The major disadvantage of autologous gene-modified tumor vaccines relates to the labor intensity and variability in gene transfer for cultures derived from primary tumor explants. The use of allogeneic vaccines certainly decreases the labor intensity and

variability of vaccine production. With this strategy, a single standardized transduced cell line or mixture of transduced cell lines is used for the vaccine. It is critical to any chance of success with a transduced allogeneic tumor vaccine that the cells share antigens with the patient's tumor. Human melanomas appear to share a number of critical antigens recognized by T cells. Whether such will be the case with other human tumors is at this point unknown. Also, a critical component of T-cell recognition is the MHC allele. Different HLA alleles will present different peptides from the same protein. For this reason, the majority of allogeneic tumor vaccines seek to match at least one HLA class I allele between the vaccine and the patient. Most commonly, the HLA allele that is matched is HLA-A2. HLA-A2 is expressed by 50% of Caucasians, and therefore an HLA-A2$^+$ allogeneic vaccine would be applicable for 50% of such patients. Interestingly, it has been recently demonstrated that the tumor cell itself does not present its own MHC class I restricted antigens during priming of the immune response during vaccination. Instead, host bone marrow-derived cells appear to take up these antigens and present them to CD8$^+$ T cells (50). If such is indeed the case, it may not be necessary that the allogeneic tumor express the same HLA allele as the patient. It is, however, critical that the allogeneic tumor line express antigens that are shared with the patient's tumor.

Virtually all of the genetically modified allogeneic tumor vaccines in current clinical trials are used for melanoma and renal cell cancer. There is a reasonable rationale for allogeneic melanoma vaccines based on the documented antigen sharing. There is certainly less documentation of antigen sharing among renal cell carcinomas. However, recent results from the autologous GM-CSF transduced renal cancer trial described above indicate that a subset of activated T cells do indeed recognize shared antigens. These findings, together with the fact that host bone marrow-derived cells appear to present tumor antigens to CD8$^+$ T cells during priming, provide the scientific rationale to explore genetically modified allogeneic vaccines clinically.

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